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DOI: https://doi.org/10.4319/lo.2013.58.3.0817

Limnol. Oceanogr., 58(3), 2013, 817–826

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Seasonal dynamics and activity of typical freshwater bacteria in brackish waters of the Gulf of Gdańsk

Kasia Piwosz,[a] Michaela M. Salcher,[b] Michael Zeder,[b] Anetta Ameryk,[a] and Jakob Pernthaler [b,*]

[a] National Marine Fisheries Research Institute, Gdynia, Poland
[b] Limnological Station, Institute of Plant Biology, University of Zurich, Kilchberg, Switzerland

Abstract

While typical freshwater and marine bacteria have been found to co-occur in brackish habitats, it is unknown if they are active members of the local bacterial assemblages or if their presence is the result of passive transport only. We followed the seasonal dynamics of typical freshwater bacteria (R-BT lineage of *Betaproteobacteria*; Ac1 *Actinobacteria*; LD12 *Alphaproteobacteria*) and of marine SAR11 *Alphaproteobacteria* in the brackish water of the Gulf of Gdańsk (southern Baltic Sea), and we assessed their incorporation of thymidine and leucine at three distinct environmental conditions. The temporal development of bacteria was driven not only by local conditions but also by phenomena resulting from the dynamic hydrology of the site. Both temperature and salinity were important factors influencing bacterial community composition, as reflected by the clear distinction of three assemblages in spring and summer and during a period of enhanced freshwater influx. During spring, high proportions of R-BT *Betaproteobacteria* and Ac1 *Actinobacteria* incorporated the radiolabeled tracers, and all three freshwater lineages were most active during the subsequent phase of low salinity. The summer period was characterized by highest abundances of Ac1 *Actinobacteria* and of both alphaproteobacterial lineages. All studied freshwater lineages were active members of the brackish water communities at specific environmental conditions, including LD12 *Alphaproteobacteria*, which have so far been considered to thrive exclusively in freshwater habitats. By contrast, the presence of the typical marine SAR11 bacteria seemed to result from passive inflow with more saline waters from the Baltic proper.

Marine and freshwater systems pose very distinct challenges for aquatic organisms. While offshore pelagic waters are often oligotrophic and with limited terrestrial effect, lakes and rivers are comparatively small, discontinuous habitats with productivity ranging from oligotropic to eutrophic and often featuring high input of terrestrial organic matter from the catchment. However, the most conspicuous difference is salinity. Marine organisms are isotonic or hypotonic, while freshwater ones are hypertonic. Both physiology and the cost of osmoregulation make the marine–freshwater boundary an insurmountable barrier for many bacteria (Logares et al. 2010). As a result, there is almost no overlap in the community composition of marine and freshwater species.

Some habitats, such as estuaries, force the mixing of marine and freshwater (micro)organisms, thus possibly favoring their coexistence. Substantial changes in the distribution and activity patterns of freshwater and marine bacteria along salinity gradients have been observed in such systems (Crump et al. 2004). Typically, the freshwater lineages rapidly diminish from saline waters, while marine are absent from freshest regions of an estuary. However, the development of truly indigenous estuarine bacterial communities is often hampered by short water residence times (Crump et al. 2004). In this context, brackish, tideless seas offer a unique opportunity to study possible adaptations and true coexistence of marine and freshwater bacteria (Riemann et al. 2008).

The Baltic Sea is among the largest brackish seas in the world. This intracontinental basin exchanges saline water with the North Sea through the narrow and shallow Belt Sea. Horizontal and vertical gradients of salinity are formed by high riverine runoff in the northern reaches, low evaporation, and restricted influx of marine waters. Salinity of the surface waters changes from 30 in Kattegat to < 1 in the Bothnian Bay, and it is in a range between 7 and 8 in the Baltic proper. Long water residence time (> 5 yr; Reissmann et al. 2009) may allow an adaptation of freshwater bacteria in the Baltic Sea to brackish conditions (Riemann et al. 2008). The Baltic Sea is severely polluted and suffers from eutrophication (Helcom 2009). Degradation of the excessive phytoplankton biomass promotes the formation of sulfidic zones below the halocline, the persistence of which is supported by weak mixing and slow exchange of the water masses.

The seasonal development of heterotrophic bacteria in the Baltic Sea depends on the region and is driven by water temperature and horizontal dynamics (Kuosa and Kivi 1989; Ameryk et al. 2005). The maxima of bacterial biomass and production follow the spring phytoplankton blooms in the northern parts (Kuosa and Kivi 1989), whereas summer is the time of highest bacterial abundance and activity in the southern Baltic proper (Ameryk et al. 2005). However, little information is available about the bacterial communities in coastal surface waters and a possible coexistence of lineages from marine and freshwater sources. A study from the Bothian Sea (Northern Baltic Sea, salinity up to 5) has shown the presence of freshwater *Actinobacteria* (Holmfeldt et al. 2009), and a coexistence of marine and freshwater lineages in waters at salinities around 7 has been reported (Herlemann et al. 2011). Nevertheless, it still remains to be shown if freshwater

* Corresponding author: pernthaler@limnol.uzh.ch

bacterial lineages are indeed metabolically active in the Baltic Sea environment and if these lineages exhibit seasonal abundance patterns that cannot be explained by passive influx only.

We compared the seasonal dynamics of several groups of freshwater bacteria (LD12 *Alphaproteobacteria, Betaproteobacteria* from the R-BT lineage, Ac1 *Actinobacteria*) with the dynamics of a typical marine group (SAR11 *Alphaproteobacteria*) in the brackish water of the Gulf of Gdańsk (southern Baltic proper). We focused on these lineages because of their high abundances and/or activity in their habitats of origin (Morris et al. 2002; Jezbera et al. 2005; Šimek et al. 2008) and because the two *Alphaproteobacteria* represent phylogenetic sibling groups from different biomes (Salcher et al. 2011). Moreover, Ac1 *Actinobacteria* and *Comamonadaceae* other than from the R-BT lineage have been previously reported from the Baltic Sea (Riemann et al. 2008; Holmfeldt et al. 2009). We also assessed the proportion of metabolically active cells in these groups during three periods of conspicuously different environmental conditions.

Methods

Sampling site—The Gulf of Gdańsk lies on the southern Baltic Sea coast; it is included in the list of the Baltic Sea Protected Areas. The time of water exchange with the open Baltic proper is around 15 days (Witek et al. 2003). The Gulf of Gdańsk is supplied with freshwater from the Vistula River, which decreases its salinity compared to the Baltic proper (from 1 at the river mouth to about 6–7 in the open basin). The maximum runoff of the Vistula River is during early spring; most of the water is transported eastward by wind-driven currents, whereas the location of the sampling site (the basin west of the river mouth) is less frequently affected (Majewski 2011). The most serious threats to the ecosystem of the Gulf of Gdańsk are the discharge of excessive nutrients and pollutants from the Vistula River, the adjacent urban agglomeration, and smaller settlements. Since the late 1990s, environmental conditions have been improving as a result of successful remediation procedures at a local level.

Samples were collected 400 m offshore at one station (54°26.87′N, 18°34.57′E) weekly from 04 April to 03 October 2007. Twenty liters of surface seawater were collected with a plastic bucket that had been thoroughly prerinsed with water from the sampling location. Temperature was measured in situ with a thermometer, and salinity was determined in the laboratory with an InoLab probe (Wissenschaftlich-Technische Werkstätten).

Chlorophyll a *and pheophytin*—Five replicates of seawater (50–300 mL each) were filtered through a glass-fiber filter (WhatmanGF/F, 47 mm). The filters were immediately frozen at −20°C and stored in the dark until further processing (< 1 month). Concentrations of chlorophyll *a* (Chl *a*) and pheophytin were determined fluorometrically after extraction in 90% high-performance-liquid-chromatography-grade acetone (Polskie Odczynniki Chemiczne).

Dissolved organic carbon and nutrients—Samples for measuring concentrations of dissolved organic carbon (DOC) and nutrients (nitrite, nitrate, ammonium, orthophosphate) were filtered through acid-cleaned (10% HCl), 0.2 μm pore size polycarbonate membrane filters (Nucleopore® Track-Etch Membrane) and stored in the dark at −20°C. DOC was determined with a TOC-5000 analyzer (Shimadzu). Nutrients were analyzed according to the recommendation of the Baltic Monitoring Program.

Nanoflagellate cell numbers—Portions of 25–50 mL of water sample (without replicates) were fixed with alkaline Lugol's solution, followed by formalin to a final concentration of 1%. Samples were then decolorized with 3% $Na_2S_2O_3$ and filtered on polycarbonate membrane filter (pore size 0.8 μm, diameter 25 mm, Isopore, Millipore). They were subsequently washed with sterile deionized water, dried and stored at −20°C until further processing. Filter sections were embedded in glycerol mountant that contained 1 μg mL^{-1} of 4′-6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). At least 200 aplastidic flagellates (based on fluorescence) per sample were counted at 1250× magnification in at least 20 microscopic fields by epifluorescence microscopy (AxioImager.M1, Carl Zeiss).

Bacterial cell numbers—Triplicate portions of each water sample (13.5 mL) were fixed with phosphate-buffered saline (PBS) paraformaldehyde (PFA; final concentration 1%) for 1 h. Ethylenediaminetetraacetic acid disodium salt (EDTA; 5 mmol L^{-1}, Sigma-Aldrich) was added to prevent aggregation of the cells during freezing. Samples were stored at −20°C until further processing. They were thawed at 4°C, and more EDTA was added to a final concentration of 10 mmol L^{-1}. Bacterial cells were stained with DAPI (1 μg mL^{-1}) in the dark for 30 min. Samples were analyzed with a InFlux™ V-GS flow cytometer (BD). The excitation sources were a ultraviolet (Lightwave Electronics, CY-PS, 60 mW, 355 nm, DAPI) and a blue laser (Coherent, Sapphire, 200 mW, 488 nm, light scatter and autotrophic cells), with detection wavelengths 460 nm for DAPI signal and 531 nm for autofluorescence of picophytoplankton. Numbers of heterotrophic cells were calculated after subtracting signals from autofluorescent cells. Two bacterial populations were distinguished based on fluorescence intensity: low-nucleic-acid (LNA) and high-nucleic-acid (HNA) bacteria.

Catalyzed reporter deposition-fluorescence in situ hybridization—Portions of 3–5 mL of samples were fixed with PBS-buffered PFA (final concentration 1%) and incubated for 1 h at room temperature in the presence of 5 mmol L^{-1} EDTA. They were then filtered (vacuum < 20 kPa) onto white, polycarbonate filters (0.2 μm pore size, diameter 47 mm, Isopore, Millipore), rinsed with distilled water, air-dried, and stored at −20°C. Catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) was performed according to the protocol by Sekar et al. (2003). We used horseradish-labeled oligonucleotide probes that target bacterial groups—all *Bacteria* (EUB I-III), *Betaproteobacteria* (Bet42a), and *Actinobacteria* (HGC69a;

Amann and Fuchs 2008)—and lineages—the R-BT subclade of the beta I cluster of *Betaproteobacteria* (R-BT065; Šimek et al. 2001), the Ac1 lineage of *Actinobacteria* (Ac1-852; Warnecke et al. 2005), and two SAR11 lineages: marine SAR11 (SAR11-441; Morris et al. 2002) and freshwater LD12 (LD12-121; Salcher et al. 2011).

Triplicate CARD-FISH preparations were evaluated on an integrated, automated high-throughput screening platform based on motorized epifluorescence microscopy and image analysis (Zeder and Pernthaler 2009). The core of the system consisted of a conventional epifluorescence microscope (AxioImager.Z1, Carl Zeiss) equipped with a motorized stage for eight microscopic slides (WSB Piezodrive 05, Carl Zeiss) and a light-emitting-diode illumination device (Colibri, Carl Zeiss).

Bacterial activity—Total bacterial activity was estimated by measuring uptake of tritiated leucine (concentration 8.3 nmol L^{-1}, specific activity 60 Ci $mmol^{-1}$, mixed 1 : 12 molar ratio with the nonradioactive form, final leucine concentration 108.3 nmol L^{-1}; Hoppe et al. 1998) and thymidine (concentration 11–12 nmol L^{-1}, specific activity 60 Ci $mmol^{-1}$; Fuhrman and Azam 1982). Triplicate 10 mL water samples were incubated for 1 h at in situ temperature and fixed with filtered formaldehyde (final concentration 1%). Blank controls (i.e., samples fixed prior to addition of the radioactive tracers) were processed in parallel. The production of new cells was calculated from thymidine incorporation rates using a conversion factor of 1.1×10^{18} cells mol^{-1} (Riemann et al. 1987).

The proportion of cells actively incorporating leucine and thymidine was estimated by microautoradiography (MAR) combined with CARD-FISH (MAR-FISH). Small subsamples (1 mL) for bacterial production were filtered onto polycarbonate filters (0.2 μm pore size, 25 mm diameter, Millipore, type GTTP), thoroughly washed with sterile distilled water, air-dried, and stored at $-20^{\circ}C$ until further processing. MAR was performed after the CARD-FISH procedure as previously described (Salcher et al. 2011). Filter sections were glued with 2% agarose (Seakem) onto coverslips with bacteria facing down, and filters were carefully peeled off after drying. Coverslips were thereafter dipped into autoradiography emulsion (NTB emulsion, Kodak) in the darkroom, placed on ice for 10 min, and exposed in the dark at $4^{\circ}C$ for 2–4 d. Subsequently, the coverslips were developed with Dektol developer (Kodak) and Kodak fixer (Kodak) following the manufacturer's instructions. Air-dried coverslips were embedded in a mounting medium containing DAPI (1 μg mL^{-1}) and evaluated with an epifluorescence microscope (Carl Zeiss). At least 100 individual cells per probe were evaluated.

Statistical procedures—Similarity analysis of the samples was based on the Bray–Curtis index, as calculated from the square root transformed abundances of the six studied populations. Such a transformation moderately reduces the effect of the most abundant bacterial groups and accentuates the influence of the less numerous ones on the results of the analysis (Clarke 1993). The statistical significance of the a priori determined grouping (i.e., Spring, Low Salinity, and Summer) was confirmed by a one-way analysis of similarities permutation test (Clarke 1993). The contribution of bacterial lineages to the observed grouping was assessed with the similarity percentage analysis. All calculations were performed using the PRIMER6 software (PRIMER-E).

Results

Physicochemical variables—Salinity was relatively constant (7.1–7.5) except for a period of 3 weeks at the beginning of June, when it decreased to 6.2–6.3 (Fig. 1A). Water temperature gradually increased during the first 2 months. The decrease in salinity coincided with a substantial temperature increase to $> 17^{\circ}C$. Thereafter, temperature ranged from $16^{\circ}C$ to $22^{\circ}C$ until the beginning of September, then it decreased to $15^{\circ}C$ (Fig. 1A). Silica concentration increased substantially between 04 and 10 July (Fig. 1B). This rise (mean value from 04 April to 04 July: 5.6 ± 1.8 μmol L^{-1}, after 10 July: 11.6 ± 3.0 μmol L^{-1}) did not coincide with the drop in salinity. DOC concentration generally varied from 1.2 to 7.6 mg L^{-1} (Fig. 1B). On 18 July, a week after the increase in silica concentration, DOC reached an exceptionally high value of 21.8 mg L^{-1}. Nitrate (NO_3^-) and ammonia (NH_4^+) also formed pronounced peaks on that day (Fig. 1C); maximal concentrations of these nutrients were observed at the last sampling date (4.1 μmol L^{-1} of nitrate and 2.5 μmol L^{-1} of ammonia) and in the case of nitrate also at the beginning of the study period (3.7 μmol L^{-1}). The concentrations of nitrite (NO_2^-) and phosphorous (PO_4^{3+}) were low (< 1 μmol L^{-1}) but gradually increased over the study period (Fig. 1C).

The sampling campaign started at the onset of the spring phytoplankton bloom, as deduced from high concentrations of Chl *a* (9.7 μg L^{-1}) and nitrate and low values of the ratio of pheophytin to Chl *a* (pheo : Chl *a* ratio; Fig. 1D). The second peak of Chl *a* (7.0 μg L^{-1}) was observed during the salinity drop. High pheo : Chl *a* ratios were observed in May after the spring bloom and in late August and September (Fig. 1D). These changes indicated a degradation of Chl *a* and phytoplankton senescence during these periods.

Bacterial seasonal dynamics—Bacterial total numbers were $< 10^6$ cells mL^{-1} until 01 June, when the temperature exceeded $15^{\circ}C$ (Fig. 2). They reached a maximum of 3.7×10^6 cells mL^{-1} on 10 July. The numbers of HNA bacteria were relatively constant, and the observed dynamics of bacteria resulted rather from changes in numbers of LNA bacteria (Fig. 2). Detection rate of all *Bacteria* with the EUB I-III probe was 52–78% of all prokaryotic cells (Fig. 2).

We observed conspicuous temporal changes in the dynamics of the investigated bacterial lineages (Figs. 3, 4A). Bacteria from the Ac1 lineage made up a substantial part of *Actinobacteria* throughout the season (17–100%, mean 58%), and represented $> 70\%$ of all *Actinobacteria* during their maximal abundance in June and July (Fig. 3A). Ac1 bacteria increased during the salinity decrease and remained numerous until August (Fig. 4A).

Betaproteobacteria of the R-BT clade increased in numbers after the phytoplankton bloom in April and

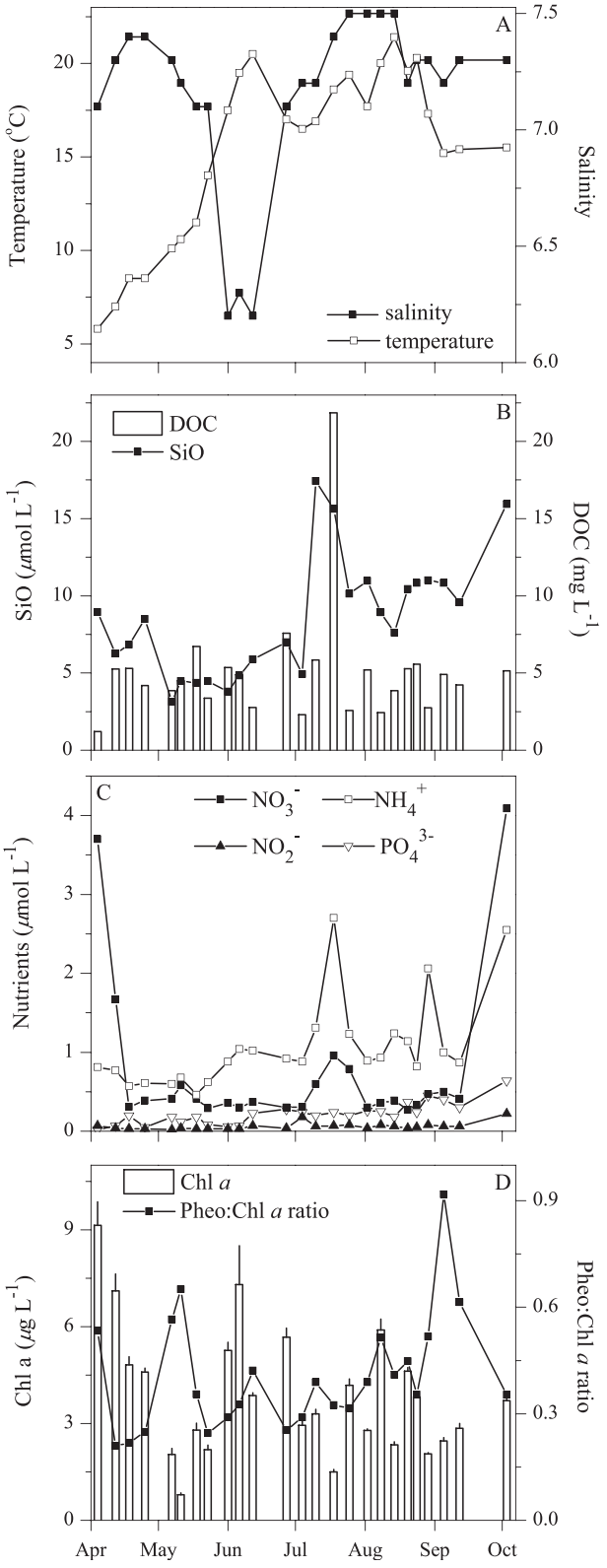

Fig. 1. Temporal dynamics of physiochemical parameters at the sampling station in the Gulf of Gdańsk in 2007: (A) temperature and salinity; (B) DOC and SiO; (C)NO_3^-, NH_4^+, NO_2^-, and PO_4^{3+}; (D) Chl *a* and pheo : Chl *a* ratio.

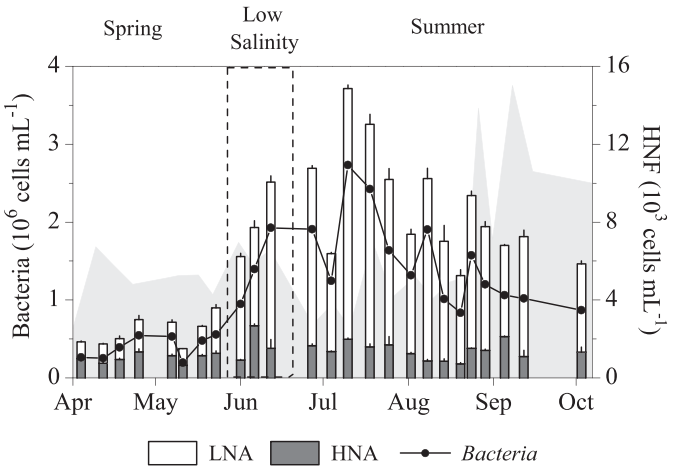

Fig. 2. Seasonal dynamics of bacteria in the Gulf of Gdańsk. Mean value from triplicate counts and standard deviation values are given for LNA and HNA bacteria. The symbols and line depict the proportions of cells hybridized with the general bacterial probe EUB I–III (*Bacteria*), and the area shaded in gray shows the numbers of heterotrophic nanoflagellates (HNF). The terms "Spring," "Low Salinity," and "Summer" correspond to the grouping of microbial assemblages as established by cluster analysis. Error bars are standard deviations of triplicate determinations.

during the event of decreased salinity in June (Fig. 4A). They constituted 8–86% of all *Betaproteobacteria* (mean 32%; Fig. 3B). Generally, the R-BT bacteria contributed the major part of *Betaproteobacteria* during periods of low abundance of the latter (early April and late August); otherwise, they were overgrown by other betaproteobacterial lineages (Fig. 4B).

Both the marine SAR11 lineage and the freshwater LD12 sister group were numerous in summer until October (Fig. 4A). Bacteria from both groups increased in numbers and proportion after the salinity rose to > 7 in late June (Fig. 3C). However, LD12 bacteria were most numerous on 10 July and gradually decreased thereafter, whereas SAR-11 showed two maxima of abundance: on 18 July and on 08 August (Fig. 4A).

Statistical analysis confirmed that the observed temporal pattern could be attributed to seasonal changes in temperature (Fig. 4B). Three assemblages could be distinguished based on changes in salinity and temperature: Spring, Low Salinity, and Summer (Fig. 4). These periods featured a significantly distinct composition of the bacterial communities with respect to the studied groups (global test: $p < 0.001$, $\rho = 0.829$). The Spring community (04 April–23 May) could be characterized by generally low numbers of all investigated bacteria, with relatively high contributions of total *Betaproteobacteria*, *Actinobacteria*, and members the R-BT lineage. During the period of Low Salinity (01–12 June), bacterial numbers were higher, and the most numerous bacteria were *Betaproteobacteria*, *Actinobacteria*, and members of the Ac1 lineage. The Summer group (27 June–03 October) was distinguished by a relatively high contribution of small (possibly LNA) bacteria related to *Actinobacteria*, the Ac1 lineage, and to the two alphaproteobacterial groups, SAR11 and LD12.

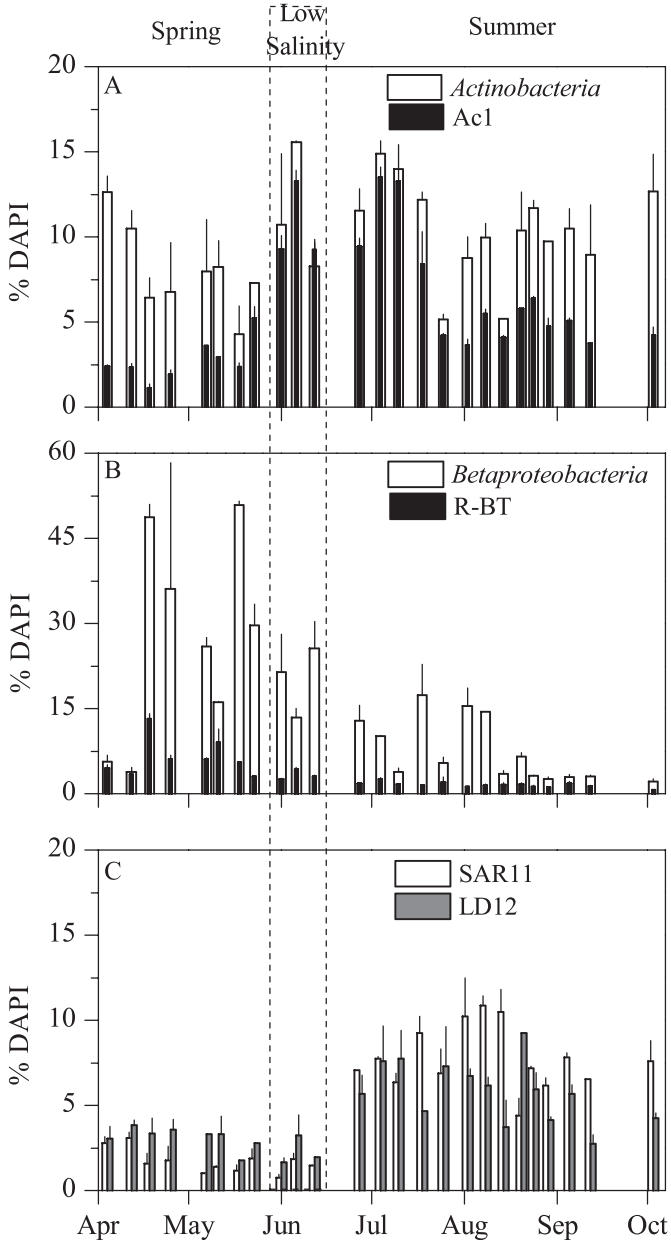

Fig. 3. Seasonal dynamics of the relative proportions (% of total DAPI cells) of (A) *Actinobacteria* and the Ac1 lineage, (B) *Betaproteobacteria* and the R-BT lineage, and (C) *Alphaproteobacteria* from the SAR11 and LD12 clades. Note the scale difference between the panels. Error bars are standard deviations of triplicate determinations.

Bacterial activity—Bacterial bulk uptake of leucine (Leu) was highest on 23 May and 06 June, and uptake of thymidine (Thy) and cell production peaked on June 06, at the time of decreased salinity (Table 1). Both the proportions and the absolute numbers of Leu-positive *Bacteria* cells increased from May to July (Figs. 5, 6). By contrast, the proportions of bacterial cells incorporating Thy decreased between these time points (Fig. 5), and the total numbers of Thy-positive cells remained relatively constant (Fig. 6).

The highest number of Leu- and Thy-positive Ac1 cells was found on 06 June during the salinity minimum (Fig. 6). During the period of decreased salinity, these bacteria were more active in the uptake of Leu and Thy than *Bacteria* in general (Fig. 5). No preference for either tracer was observed in this group.

The proportion of Thy-positive *Betaproteobacteria* was always higher than of *Bacteria* (Fig. 5), and their total numbers did not change between the sampling times (Fig. 6). In contrast, the numbers of Leu-positive *Betaproteobacteria* were relatively low on 23 May and 06 June as compared to 18 July. A preference for Thy was observed (Fig. 5). The highest proportion of Thy-positive R-BT bacteria ($> 50\%$) was observed in May (Fig. 5), whereas it did not exceed 30% of the total population at the other sampling dates. The proportion of Leu-positive R-BT bacteria was always $> 50\%$ (Fig. 5). These bacteria were generally more active in Leu uptake than *Bacteria*. The highest number of both Thy- and Leu-positive R-BT bacteria was observed on June 06 (Fig. 6). At this date, they constituted approximately three-quarters of all Leu-positive *Betaproteobacteria*.

LD12 bacteria showed little uptake of leucine. Thy-positive cells were low in May, but their proportions and numbers increased on 06 June and 18 July (Figs. 5, 6). Only small proportions of SAR11 bacteria incorporated either tracer (Fig. 5). However, despite the low proportions of active cells, the number of Leu-positive SAR11 bacteria was comparatively high on 18 July due to their high total abundances at that date (Figs. 4A, 6).

Discussion

Seasonality and dynamics of bacteria in the Gulf of Gdańsk—The eutrophic coastal waters of the of the Gulf of Gdańsk support rich phytoplankton communities during the productive season between April and October (Witek et al. 1997). Exudates released by phytoplankton and organic matter of riverine origin supply heterotrophic bacteria, whose production in summer may reach up to 80% of gross primary production (Ameryk et al. 2005). The observed seasonal dynamics of the total bacterial numbers in the Gulf of Gdańsk, assessed at high temporal resolution, could be attributed mainly to presumably small bacteria with low nucleic acid content (Fig. 2). This view is supported by a comparison with the dynamics of the studied FISH-defined populations. Specifically, the highest fluctuations in numbers were observed in Ac1 *Actinobacteria*, LD12, and SAR11 *Alphaproteobacteria* (Fig. 4) that likely contributed to LNA bacteria due to their typically small cell sizes (Morris et al. 2002; Salcher et al. 2011). By contrast, large-sized bacteria R-BT *Betaproteobacteria* that likely contributed to HNA bacteria changed little during this period.

LNA bacteria have been considered less active members of bacterial communities (Gasol et al. 1999), but they may in fact feature higher biomass-specific growth rates than larger HNA bacteria (Salcher et al. 2010). Moreover, these bacteria may also profit from partial protection against size-selective protistan predation (Pernthaler 2005). In view of the high numbers of aplastidic nanoflagellates in the

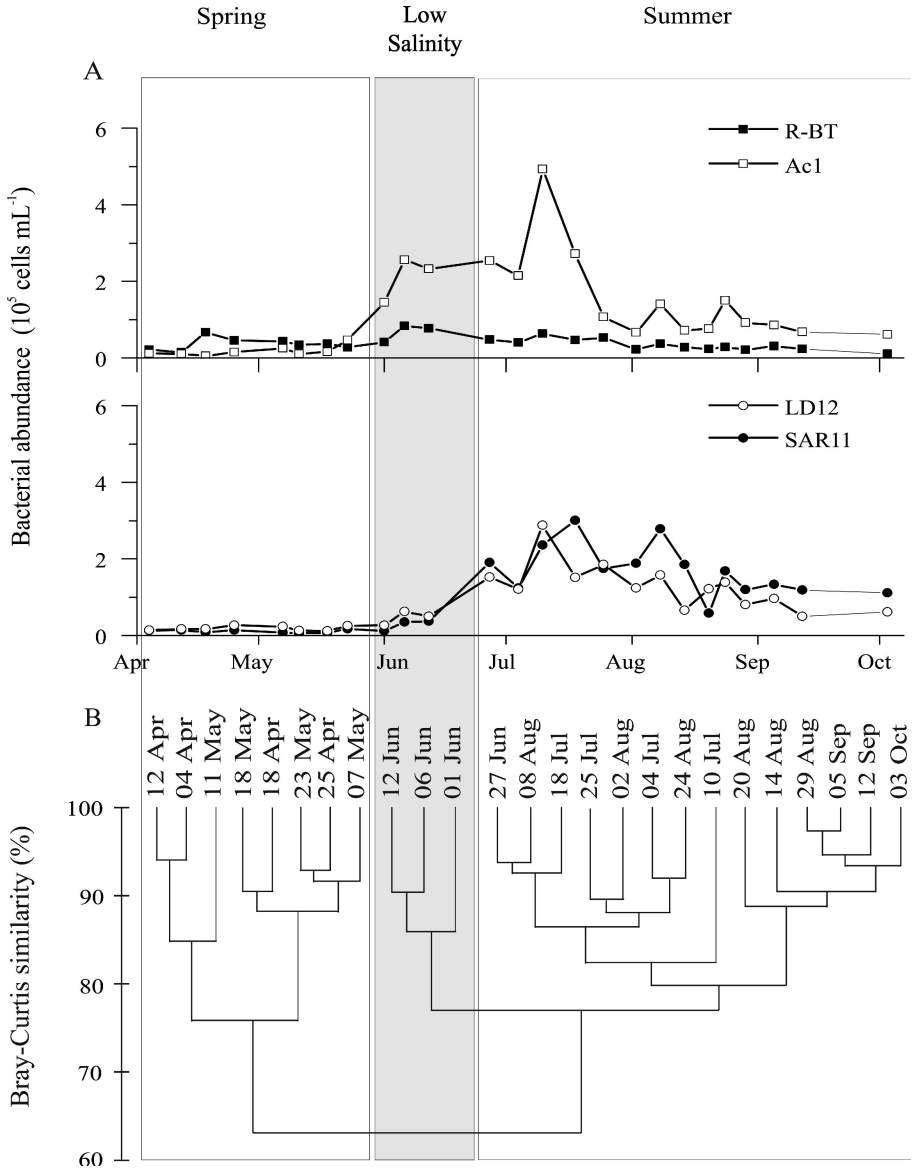

Fig. 4. (A) Seasonal dynamics of the R-BT, Ac1, LD12, and SAR11 bacteria; (B) dendrogram of hierarchical clustering (Bray–Curtis similarity, group-average linking, square-root-transformed abundance data). The three depicted groups (“Spring,” “Low Salinity,” and “Summer”) differ significantly in terms of bacterial assemblages (global test: $p < 0.001$, $\rho = 0.829$).

Gulf of Gdańsk, grazing pressure on bacterioplankton might have been substantial (Fig. 2). Although not all nanoflagellates in this habitat were found to be bacterivores, numerous mixotrophs also contributed to the total bacterivory (Piwosz and Pernthaler 2010).

The observed seasonal dynamics of the investigated bacterial groups was much higher than of total *Bacteria* (Figs. 2, 4). Two of the three studied typical freshwater lineages in the Gulf of Gdańsk (but not Ac1 *Actinobacteria*) showed seasonal abundance patterns that resembled the temporal development of these bacteria in lacustrine habitats: members of the R-BT lineage increased after the spring phytoplankton bloom (Salcher et al. 2008; Šimek et al. 2008), and LD12 bacteria were most numerous in summer (Salcher et al. 2011). Moreover, the temporal dynamics of SAR11 also corresponded to observations from marine sites (Carlson et al. 2008).This suggests that their respective temporal niches in brackish water might be similar to those in their habitats of origin and hence that they might be controlled by comparable sets of environmental variables.

However, the Gulf of Gdańsk is a much more dynamic system than freshwater lakes. The short water residence time of approximately 15 d (Witek et al. 2003) may prevent formation of unique brackish bacterial communities (Crump et al. 2004). Salinity values of > 7 for most of the study period would indicate a predominant affect of the Baltic proper on the observed patterns in bacterial dynamics in the Gulf of Gdańsk but in turn would not account for the persistence of freshwater bacteria. Moreover, the short-term decrease in salinity to 6 caused

Table 1. Incorporation rate (mean ± SD of triplicate measurements) of leucine and thymidine (pmol Leu or Thy L^{-1} h^{-1}) by the total bacterial community and estimated numbers of newly produced cells (NPC; 10^6 cells mL^{-1} d^{-1}).

	23 May	06 June	18 July
Leucine	368.6±12.5	361.5±96.6	131.0±15.8
Thymidine	53.1±4.0	185.7±5.8	37.4±3.8
NPC	1.40±0.10	4.90±0.14	0.99±0.10

significant changes in the investigated components of bacterial community (Fig. 4B). This agrees with observations from estuaries, where salinity ranges between 6 and 7 induced the most conspicuous changes in the composition of bacterial assemblages (Crump et al. 2004). A decrease in salinity of about 1 in the Gulf of Gdańsk corresponds to only approximately 13% of additional freshwater input. Since the abundance changes of the studied bacterial populations during that period were much more substantial (up to threefold of their previous densities), the observed community shift could not have been caused solely by passive transport of riverine bacteria. The same conclusion is reached if estimates of newly formed cells per day, as deduced from bulk thymidine incorporation rates (Bell 1990), are compared to the theoretical cell import rates due to water exchange (4.9×10^6 newly produced cells mL^{-1} d^{-1} vs. $\sim 6.5 \times 10^5$ advected cells mL^{-1}, corresponding to 13% of the bacterial numbers in the Vistula River; Table 1). In

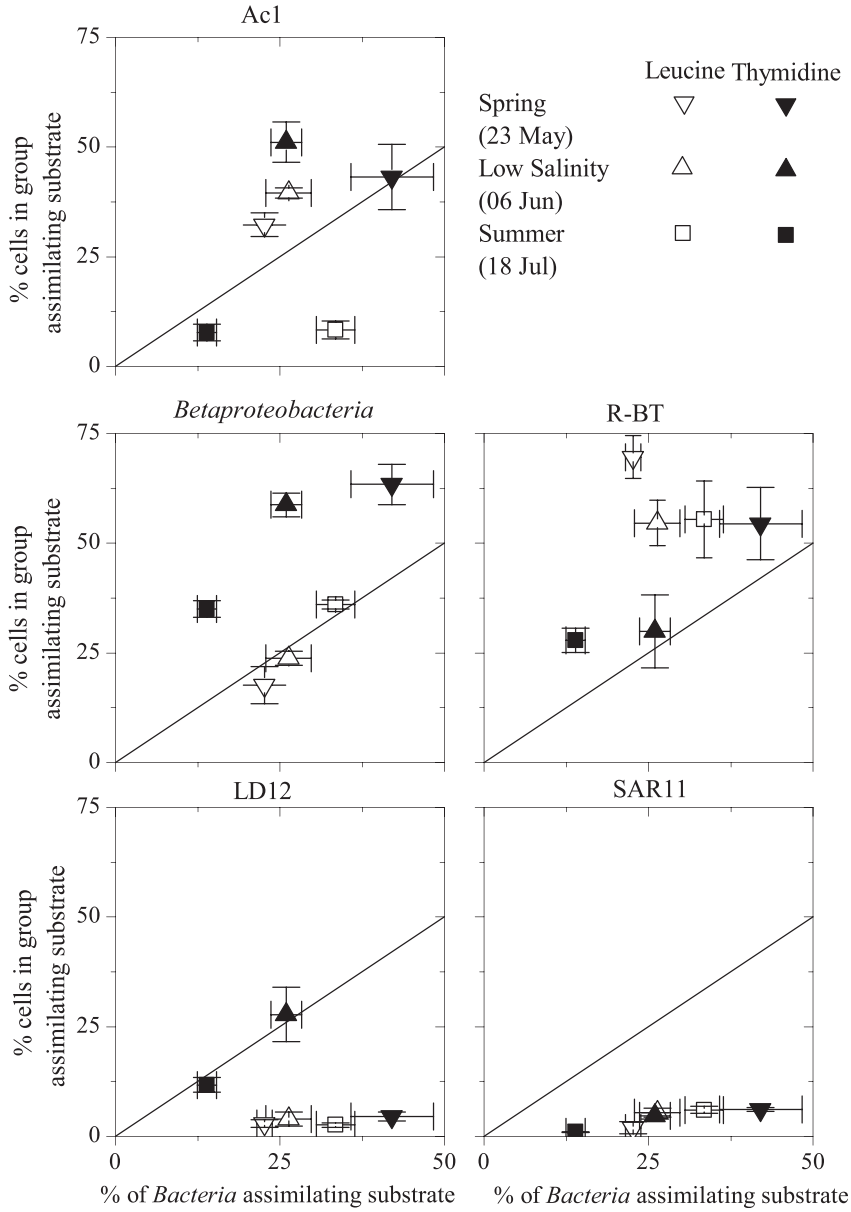

Fig. 5. Uptake of Leu and Thy by the studied bacterial groups. Points above or below the 1 : 1 line indicate higher and lower proportions of active bacteria than the community average (i.e., compared to all *Bacteria* as detected with the general probe EUB I–III). Error bars are standard deviations of triplicate determinations.

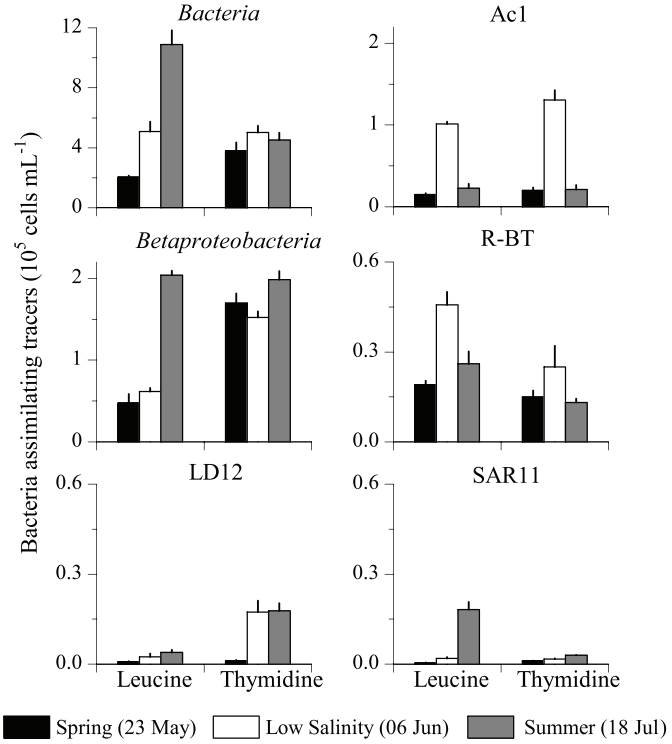

Fig. 6. Numbers of cells from different bacterial taxa that took up Leu and Thy on three dates during the study. Error bars are standard deviations of triplicate determinations.

addition, we also noted a conspicuous increase in the uptake of Leu and/or Thy by freshwater bacteria (Ac1, R-BT) at that time (Figs. 5, 6). Thus, although the advection of water masses was undoubtedly an important factor for shaping bacterial communities in the Gulf of Gdańsk, the observed population dynamics were likely also driven by an active response of some bacterial taxa to changing growth conditions.

Freshwater bacterial lineages in brackish waters—The presence of freshwater bacteria in estuarine and coastal habitats is well documented (Crump et al. 2004). Although a direct comparison with information on estuarine bacteria from Chesapeake and Delaware Bays provided by the Global Ocean Sampling (GOS) project (Rusch et al. 2007) is hampered by terminology (i.e., it is unclear if "Frankineae" and "*Comamonadaceae*" indeed correspond to Ac1 *Actinobacteria* and R-BT *Betaproteobacteria*), a SILVA database search confirmed that the GOS data set included genotypes from all three studied freshwater groups. The occurrence of bacteria from lacustrine lineages in the Baltic Sea has also been documented (Riemann et al. 2008; Holmfeldt et al. 2009). Our study extends these findings by providing information about the temporal dynamics of such bacteria. We furthermore assessed if freshwater bacteria are indeed a metabolically active component of brackish water microbial assemblages and if their activity is affected by intrinsic and extrinsic factors, such as phytoplankton blooms and variable influx of freshwater.

The Ac1 clade of *Actinobacteria* is a phylogenetically diverse, ubiquitous, freshwater lineage (Newton et al. 2007). They typically form maxima of abundance in spring (May–June) and fall (October–December; Allgaier and Grossart 2006). Similar seasonality was also observed in the northern basins of the Baltic Sea (Holmfeldt et al. 2009). The highest proportions of Leu- or Thy-positive Ac1 cells in the Gulf of Gdańsk was observed in May and during the salinity drop in June (Figs. 5, 6). However, only the latter situation was accompanied by an increase in cell numbers, and the maximal numbers of active Ac1 cells were observed in July and August (Fig. 3A). This discrepancy between the numbers and proportions of active cells might reflect the high resistance of small actinobacterial cells to predation mortality, resulting in the accumulation of slowly growing albeit largely inedible cells (Pernthaler 2005).

Betaproteobacteria and the betaproteobacterial R-BT lineage are important members of bacterial communities in lakes (Salcher et al. 2008). Their elevated numbers and proportion after the spring phytoplankton bloom (Figs. 3A, 4) and efficient incorporation of leucine (Fig. 5) indicate that they were able to utilize autochthonous, algal-derived organic matter (Šimek et al. 2008). Members of the R-BT clade were found to be active in the brackish compartment of a subtropical Lagoon (Alonso et al. 2009). In the Gulf of Gdańsk, where salinity is higher, these bacteria were three times less numerous but also three times more active. They were particularly stimulated during the period of decreased salinity (Fig. 6), when they contributed to 75% of Leu-positive *Betaproteobacteria*. The low numbers but high activity may indicate that R-BT bacteria were tightly controlled by mortality, such as grazing by protists or viral lysis (Cottrell and Kirchman 2004; Jezbera et al. 2005). This conclusion is also supported by the presence of large aggregates of R-BT bacteria in the samples (not shown), which has been interpreted as an antipredation strategy of some bacteria (Pernthaler 2005). So far, two *Limnohabitans* spp. strains targeted by probe R-BT have been cultured (Kasalicky et al. 2010). These isolates do not grow at NaCl concentrations $> 0.5\%$, which might suggest the presence of novel *Limnohabitans* ecotypes or even species in brackish habitats that are able to adapt to the higher levels of salinity in the Gulf of Gdańsk (0.6–0.7%).

Coexistence of closely related marine and freshwater bacteria—SAR11 and LD12 are phylogenetically closely related sister lineages that are generally believed to occur exclusively in separate biomes. (Logares et al. 2010). SAR11 bacteria are often a numerically dominant component of prokaryotic communities in marine waters (Morris et al. 2002), and there is one lineage of SAR11 that is found mainly in brackish waters (Logares et al. 2010). By contrast, LD12 bacteria are an important member of epilimnetic bacterial communities in temperate lakes ($> 30\%$ of DAPI counts and $> 50\%$ of active *Bacteria*) in summer at water temperatures $> 16^\circ$C (Salcher et al. 2011). The coexistence of brackish and marine SAR11 bacteria in the Baltic Sea during early summer (June–July) has been recently confirmed (Herlemann et al. 2011). However, that study did not encounter representatives of the freshwater LD12 lineage even in the freshest reaches of the Baltic Sea (at salinities < 1).

Our study for the first time shows that freshwater LD12 bacteria are abundant and moreover metabolically active in a brackish water habitat both during periods of freshwater influx and during summer. Interestingly, we found relatively high proportions of LD12 bacteria that assimilated thymidine (up to $> 25\%$; Fig. 5), a compound that was selected against in lakes (Salcher et al. 2011). Nevertheless, the observed thymidine uptake clearly suggests that LD12 bacteria remained active, potentially deoxyribonucleic acid (DNA)-synthetizing members of the bacterial community on transport from riverine sources into the Gulf of Gdańsk.

Our results critically depend on the applied methodology. We assessed the presence and activity of the marine and freshwater (LD12) lineages using the oligonucleotide probes SAR11-441 (Morris et al. 2002) and LD12-121 (Salcher et al. 2011). Since both of these probes feature at least two strong base mismatches with nontarget sequences from the other lineage (assessed with the SILVA database, release 107; Pruesse et al. 2007), it can be assumed that they are specific for their respective target groups. We did not monitor the presence of the brackish lineage because no specific FISH probe was available.

The proportion of SAR11 and LD12 bacteria in the Gulf of Gdańsk was up to 10% of DAPI counts, which corresponded to maximally 3×10^5 cells mL^{-1} (Figs. 3A, 4). This proportion is two- to threefold less than in their respective core habitats (Morris et al. 2002; Salcher et al. 2011). This difference would imply a maximal diel exchange of approximately 20–30% of the standing stock population of SAR11 by influx from the Baltic proper but considerably less for LD12 due to lower freshwater input ($\sim$ 1% daily; Witek et al. 2003). The two lineages share many features (summarized in Salcher et al. 2011), such as small cell size and seasonal maxima in the euphotic zone during summer. SAR11 have been shown to actively assimilate leucine (Malmstrom et al. 2005). The proportion of Leu-active SAR 11 cells was generally very low, although their total numbers increased on 18 July. This low activity may indicate that the marine SAR11 bacteria, probably introduced from the open Baltic proper, were not very active at such low salinity. However, the success of the oligotrophic SAR11 bacteria could also be hampered by the high eutrophic status of the Gulf of Gdańsk. All together, it appears that bacteria from the freshwater LD12 lineage could adapt more readily to likely suboptimal levels of salinity than their marine counterpart.

In this study we showed typical freshwater bacteria (R-BT lineage of *Betaproteobacteria*, Ac1 *Actinobacteria*, LD12 *Alphaproteobacteria*) to be active members of brackish bacterial communities. The temporal development of these bacteria was driven both by local conditions and by the dynamic hydrology of the Gulf of Gdańsk. Both temperature and salinity were important factors influencing bacterial community composition, as reflected by the clear distinction of three assemblages in spring, in summer, and during a period of enhanced freshwater influx. High numbers of newly produced cells and proportions of Leu- and/or Thy-positive cells in populations of the investigated freshwater bacterial lineages indicate that the observed temporal dynamics resulted from their active growth. By contrast, the presence of the typical marine SAR11 bacteria seemed to result from passive inflow with more saline waters from the Baltic proper.

Acknowledgments

We thank Hanna Wróblewska and Marianna Pastuszak for nutrient analysis and Mariusz Zalewski for his assistance during measurements of Chl *a*. The Foundation for Hydrobiology and Limnology Zurich and the University of Zurich are gratefully acknowledged for supporting K.P. during her stays in Switzerland. This study was supported by the Polish Ministry of Science and Higher Education, projects N304 032 32/1616 and 795/N-CBOL/2010/0; by the Swiss National Fund, project 31EE30-132771, as part of Collaborative Research Program (EUROCORES) Ecological and Evolutionary Functional Genomics (EuroEEGF); and by the Swiss Federal Department of Foreign Affairs through Polish-Swiss Research Program, project PSPB-036/2010: Diversity and Ecology of Mixotrophic Nanoflagellates in the Gulf of Gdańsk (DEMONA).

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Associate editor: Wade H. Jeffrey

Received: 16 July 2012
Accepted: 19 December 2012
Amended: 22 January 2013